Original Article
Genotoxicity assessment in autism spectrum disorder patients using sister chromatid exchange and chromosomal aberration assays

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Abstract: Autism spectrum disorder (ASD) is one of the most common childhood neurodevelopmental disorders. ASD is characterized by impairment in social, behavioral, and communicative functions. ASD has a variable presentation and its etiology is still poorly understood. Several studies have shown that oxidative stress plays a vital role in the pathology of ASD. Oxidative stress causes severe damage to biomolecules, especially DNA. In this study we investigated the genotoxicity in lymphocytes of ASD patients, using sister chromatid exchange (SCE) and chromosomal aberration (CA) assays. Eighteen ASD subjects and age-matched healthy controls were recruited in the study. The results showed that ASD is associated with a significant increase in the frequencies of SCEs and CAs ($P < 0.01$, $P < 0.0001$, respectively) in lymphocytes derived from patients with ASD. Our results indicate that increased chromosomal instability could play an important role in the etiology of ASD.

Keywords: Autism spectrum disorder (ASD), DNA damage, oxidative stress, genotoxicity, sister chromatid exchange assay, chromosomal aberration assay

Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by impairment in social, behavioral, and communicative functions [1]. The prevalence of ASD has dramatically increased to approximately 1-2% of children worldwide [2]. This could largely be attributed to changes in diagnostic practices, referral patterns, availability of services, younger age at diagnosis, and improved public awareness [3]. Males are at a higher risk for ASD than females with a ratio of 4:1 [1, 4]. Although there is still no general consensus on the etiology of autism [5], emerging evidence suggests a potential role of oxidative stress in the development of ASD [6-9].

The most significant consequence of oxidative stress is related to DNA damage [10]. Oxidative stress increases the accumulation of sister chromatid exchanges (SCE) and chromosomal aberrations (CA), both of which are indicative of genotoxicity. Furthermore, free radicals can result in DNA single-strand breaks (SSBs), double-strand breaks (DSBs), and other non-DSB lesions [10, 11].

Oxidative stress biomarkers have been examined among autism patients in several studies. Most of these studies have revealed consistent findings in which the patients have elevated reactive oxygen species (ROS) and reduced antioxidant levels [8, 12-17]. It has also been reported in various autism disease models that oxidative stress exerts an effect on the central nervous system (CNS) [18]. Furthermore, neuroligin-deficient mutants of *Caenorhabditis elegans* showed an increased basal level of oxidative stress accompanied with a higher level of oxidized proteins [18, 19]. More importantly, a valproic acid (VPA)-induced ASD mouse model

Table 1. Frequency of SCEs in ASD patients and normal controls

Controls			ASD Patients		
Control ID	Age	SCE/cell	Patient ID	Age	SCE/cell
1	1.5	3.44	1	2.4	3.68
2	3	2.84	2	2.5	6.42
3	3	7.12	3	4	5.76
4	3.5	3.96	4	4.6	6.73
5	4	4.56	5	4.8	6.20
6	4	6.92	6	4.9	7.08
7	4	6.36	7	5.6	5.6
8	4	4.48	8	5.6	6.32
9	5	6.04	9	5.9	5.64
10	7.5	3	10	6	5.52
11	8	5.8	11	6	5.89
12	8	3.24	12	6.3	6.72
13	8	3.04	13	6.4	5.76
14	8	6.48	14	6.5	5.28
15	8	5.5	15	6.6	5.48
16	9	2.92	16	7.1	8.04
17	9	3.68	17	10.8	6.96
18	10	3.64	18	10.9	7.36
Mean ± S.E.	5.97 ± 0.62	4.61 ± 0.36	Mean ± S.E.	5.93 ± 0.52	6.14 ± 0.23

Age is in years, SCE: sister chromatid exchange, S.E.: standard error of the mean.

demonstrated elevated levels of malondialdehyde and nitrite [20].

To the best of our knowledge, genotoxicity assessment using sister SCE and CA assays has not been investigated before on ASD patient-derived cells. Accordingly, in this study, we investigate the presence of genotoxic lesions in lymphocytes derived from a representative group of autistic children.

Materials and methods

Subjects

Eighteen children with ASD in Jordan were recruited for this study with their guardians' informed consent. Additionally, a control group of eighteen age-matched healthy children were also recruited for the study (**Table 1**). Subjects taking medications or any other supplements for at least 3 months prior to the blood sample collection were excluded from participating in this study. The study was approved by the Institutional Review Board (IRB) of the Jordan University Hospital (JUH), (number 10/2016/4739). All blood samples were collected after receiving a written informed consent.

Cell cultures

A sample of 5 ml venous blood was collected from each subject under sterile conditions in heparin tubes. Blood cultures were set up by inoculating 1 ml of freshly withdrawn blood into 25 ml tissue-culture flasks containing 9 ml of complete karyotyping media (chromosome medium P, Euro-Clone, Italy, cat #: EKA-MTB-100). The cultures were incubated in the dark for 69-72 hours in a 5% CO_2 incubator at 37°C. In the last 1.5 h of the incubation period, colcemid (EuroClone, Italy, cat #: ECM-0040B) was added directly to the cultures (final concentration 0.1 µg/ml) to arrest the lymphocytes at metaphase.

Sister chromatid exchange assay

For the differential staining of the sister-chromatids, 5-bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO, USA, cat #: B5002) was added to the cultures (final concentration: 25 µg/mL) prior to incubating the cultures. All cultures were maintained in the dark to minimize any photolysis of the BrdU. After that, cultured lymphocytes were harvested using a hypotonic solution (0.075 M KCL, EuroClone, Italy, cat #: ECM0543) and fixed in (3:1) methanol: glacial acetic acid solution as previously described [21-23]. The slides were allowed to air dry, then aged for 24 h in the dark and then differentially stained using a florescence-plus-Giemsa technique as previously described [23]. To score SCEs, twenty-five clearly differentiated second metaphases that contained between 42 and 46 chromosomes were analyzed for each subject.
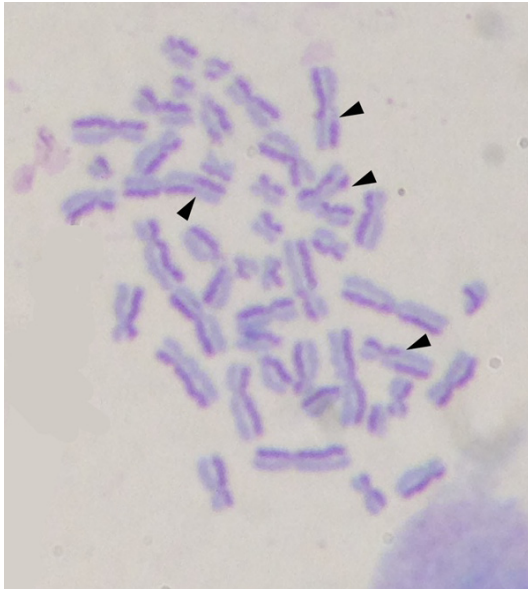
Figure 1. Representative metaphase spread with differentially stained sister chromatids (M2) from lymphocytes of a patient with autism spectrum disorder (ASD). Examples of sister chromatid exchanges (SCE) are indicated by the arrows.
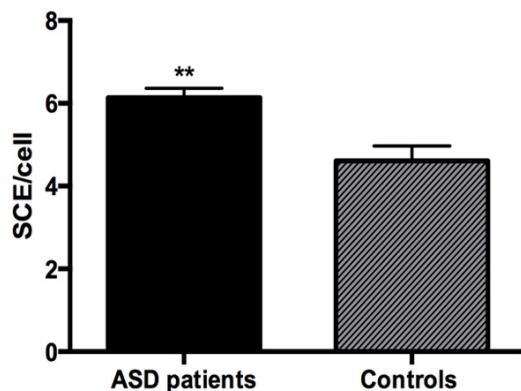

Figure 2. Average of sister chromatid exchanges (SCEs)/cell in autism spectrum disorder (ASD) patients and control group. SCEs were examined in the M2 cells of cultured blood lymphocytes. The data are expressed as the mean ± S.E. Significant difference observed between the two groups (Kolmogorov-Smirnov comparison test, n = 18/group; P < 0.01, *P* value summary: **).

Chromosomal aberration assay

Air-dried slides prepared from lymphocytes cultures without BrdU were stained with 5% Giemsa stain prepared in Gurr buffer (Gibco, Invitrogen, UK, cat #: 10582013.). Structural CAs were evaluated in 50 well spread metaphases per subject. CA was classified accord-

ing to the International System for Human Cytogenetic Nomenclature (ISCN) into chromatid breaks and chromosomal breaks and exchanges [24, 25].

Cell kinetics analysis

The mitotic index (MI) was calculated by scoring at least 1000 cells for each subject and dividing the number of cells that were in the metaphase stage by the total number of cells scored. The cell proliferation index (PI) was calculated by scoring a total of at least 100 metaphase cells from each subject using the following formula: PI = (M1 × 1) + (M2 × 2) + (M3 × 3)/(M1 + M2 + M3). M1, M2 and M3 are the number of cells undergoing the first, second and third mitosis, respectively, during the 72 h of the cell culture incubation period. MI was used as an indicator of the status of the G2 stage progress in the cell cycle, whereas the PI reflects S and G2 stages progress [26].

Statistical analysis

Graphpad Prism Software (version 6, La Jolla, CA, USA) was used for statistical analysis. Data were expressed as the mean ± standard error (S.E.). The comparisons of parameters were performed using Student's *t*-test. Correlation statistics were then performed using Kolmogorove-Smirnov correlation test. The differences were considered significant at *P* < 0.05.

Results

Eighteen ASD patients and 18 healthy controls, with a mean age of 5.93 ± 0.52 years and 5.97 ± 0.62 years, respectively, were recruited in this study (**Table 1**). Next, we collected blood samples from the participating subjects and established lymphocyte cell cultures *in vitro*. To investigate the presence of genotoxic stress, we ran both CA and SCE assays on the patient-derived lymphocytes of both the ASD patients and the healthy subjects. In order to score SCEs, a total of 900 (25 per subject) differentially stained M2 metaphase cells that had between 42 and 46 chromosomes were visualized and examined using light microscopy (**Figure 1**). There was a 33% significant increase in the frequency of SCEs in the ASD patients compared to the control group (6.14 ± 0.23 and 4.61 ± 0.36 respectively, *P* < 0.01) (**Table 1; Figure 2**).
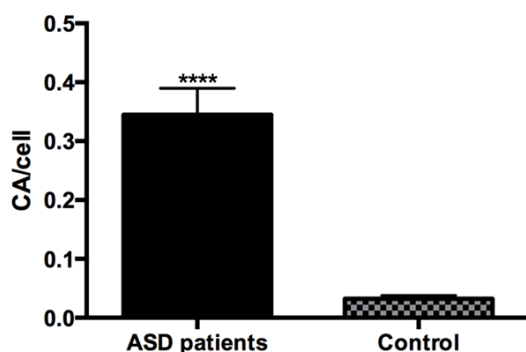
Figure 3. Levels of chromosomal aberrations (CAs) in lymphocytes from autism spectrum disorder (ASD) patients and healthy controls. CAs were examined in metaphase cells of cultured blood lymphocytes. The data are expressed as the mean ± S.E. Breaks and exchanges were included in the aberrations assessment. CAs were significantly increased in ASD patients compared with controls (Kolmogorov-Smirnov comparison test, n = 18/group; $P < 0.0001$, p value summary: ****).

The previous results were confirmed by the CA assay. CA data was obtained through the analysis of a total of 1800 metaphases (50 per subject) in both the ASD and normal subjects. Interestingly, a novel increase of 12-times higher in the level of structural CAs was observed in the ASD patients in comparison to the control group (0.35 ± 0.05 and 0.03 ± 0.01 respectively, $P < 0.0001$) (**Figure 3**; **Table 2**). A representative example for chromosomal aberrations that have been identified in ASD patient-derived lymphocytes is shown in **Figure 4**.

Finally, MI and PI were used as indicators of blood lymphocyte cytotoxicity. There was no significant difference in MI between the two groups ($P > 0.05$). However, the PI was significantly lower in the ASD patients (2.51 ± 0.03) when compared to the control group (2.68 ± 0.03) $P < 0.01$ (**Table 3**).

Discussion

ASD is a behavioral disorder that has variable causes and clinical pictures [9]. To date, the exact cause behind ASD is not well-understood [5, 9]. Current evidence suggests that the pathogenesis of ASD is profoundly complex and includes genetic, environmental and immunological factors [5, 9, 27, 28].

Oxidative stress occurs as a result of an imbalance between the production of reactive oxygen species (ROS) and endogenous antioxi-

dants in living organisms [29]. Oxidative stress has been implicated in the pathogenesis of major psychiatric disorders due to the higher vulnerability of brain tissue to oxidative damage [8].

Several studies have reported that autistic patients suffer from increased oxidative stress [15, 30]. Furthermore, high levels of lipid peroxidation, urine 8-hydroxy-2-deoxyguanosine, and other oxidants have all been reported in autistic patients [6, 31]. On the other hand, ASD patients have decreased plasma activity of glutathione peroxidase [32], reduced levels of total glutathione, a lower redox ratio of reduced glutathione to oxidized glutathione (GSSG) in plasma, as well as decreased catalase and superoxide dismutase (SOD) activity in erythrocytes [33-36]. Interestingly, the antioxidants N-acetyl-cysteine [37], coenzyme Q10 [38] and ascorbic acid [39] ameliorate symptoms in autistic patients, possibly by reducing oxidative stress.

In agreement with the elevated levels of oxidative stress, high levels of DNA damage have been detected in ASD patients using the comet assay [40]. In this study we aimed to further investigate the consequence DNA damage. Therefore, both CA and SCE assays were performed to assess the extent of genotoxic stress in the lymphocytes of ASD patients. These assays are considered highly sensitive and predictive of DNA damage due to exogenous or endogenous factors such as oxidative stress [21, 41, 42].

CA and SCE can occur spontaneously, but they increase significantly as a result of exposure to genotoxic stimuli [41, 43, 44]. In the present study, a significant elevation of SCE and CA has been revealed in autism patients' chromosomes. This abnormal increase indicates that the pathological ROS upregulation in ASD patients is sufficient to disrupt the genomic integrity. SCE occurs as a result of DNA DSB repair mechanism by homologous recombination (HR) between sister chromatids [48]. Therefore, elevated levels of SCEs are indicative of DNA damage and subsequent repair. The SCEs rate was 33% a higher in ASD patients than in the controls, which reflects higher rate of DNA damage by DSBs. Moreover, the CA frequency for ASD patients was 12 times more than the controls' CAs.

Table 2. Frequency of CAs in ASD patients and normal controls

Controls					ASD patients				
	Aberrations		Total aberrations	CA/cell		Aberrations		Total aberrations	CA/cell
Control ID	Chromatid type	Chromosome type			Patient ID	Chromatid type	Chromosome type		
1	0	0	0	0	1	5	11	16	0.32
2	0	3	3	0.06	2	3	11	14	0.28
3	1	2	3	0.06	3	2	6	8	0.16
4	0	1	1	0.02	4	9	16	25	0.5
5	0	1	1	0.02	5	21	18	39	0.39
6	0	0	0	0	6	2	15	17	0.34
7	0	3	3	0.06	7	1	6	7	0.14
8	0	1	1	0.02	8	8	16	24	0.48
9	0	1	1	0.02	9	3	6	9	0.18
10	0	3	3	0.06	10	9	31	40	0.8
11	0	3	3	0.06	11	2	13	15	0.3
12	0	2	2	0.04	12	5	14	19	0.38
13	0	0	0	0	13	4	11	15	0.3
14	0	0	0	0	14	2	13	15	0.3
15	0	2	2	0.04	15	3	5	8	0.16
16	0	1	1	0.02	16	0	7	7	0.14
17	0	3	3	0.06	17	9	29	38	0.76
18	0	2	2	0.04	18	2	12	14	0.28
Mean ± S.E.	0.05 ± 0.05	1.39 ± 0.27	1.61 ± 0.29	0.03 ± 0.01	Mean ± S.E.	6 ± 1.49	15.7 ± 2.99	21.7 ± 4.22	0.35 ± 0.05

CA: chromosomal aberration, S.E.: standard error of the mean.

Figure 4. Example of metaphase with dicentric chromosome (indicated by the arrow) from an autism spectrum disorder (ASD) patient.

To the best of our knowledge, this is the first study to examine the genotoxicity associated with ASD using SCE and CA. The results obtained here are in agreement with the previous studies. It confirms that ASD is associated with oxidative stress as indicated with genotox-

Table 3. Mitotic index and proliferation index of autistic patients and normal controls

	Controls	ASD patients
MI%: (mean ± S.E.)	5.09 ± 0.23	5.17 ± 0.38
PI: (mean ± S.E.)	2.68 ± 0.03	2.51 ± 0.03*

*Significant difference from control group ($P < 0.05$), MI: mitotic index, PI: proliferation index, S.E.: standard error of the mean.

icity. In addition, this study validates the SCE and CA assays as sensitive methods for genotoxicity investigation. Collectively, our findings suggest that the increase in DNA damage detected in the patient-derived lymphocytes of ASD patients can be a consequence of the excess production of free radicals. Oxidative stress and the resulting chromosomal instability could contribute to the development of ASD.

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Disclosure of conflict of interest

None.

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